1-(3′-[^{125}I]Iodophenyl)-3-methy-2-pyrazolin-5-one: Preparation, Solution Stability, and Biodistribution in Normal Mice

Yuuhei Sano, Tomokazu Motomura, Fumihiko Yamamoto, Miki Fukuda, Takahiro Mukai,* and Minoru Maeda*

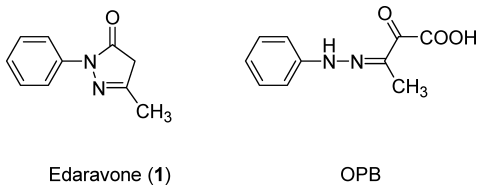
Graduate School of Pharmaceutical Sciences, Kyushu University; 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan. Received February 1, 2010; accepted May 15, 2010; published online May 18, 2010

3-Methyl-1-phenyl-2-pyrazolin-5-one (edaravone, 1), known as a potent free radical scavenger, has been developed as a medical drug for the treatment of acute cerebral infarction. With the aim of developing radiotracers for imaging free radicals *in vivo*, 1-(3′-[^{125}I]iodophenyl)-3-methy-2-pyrazolin-5-one (^{125}I-2) was synthesized by two methods, *via* isotopic exchange and interhalogen exchange under solvent-free conditions, in which iodo- and bromo-derivatives were used as labeling precursors, respectively. After HPLC purification, ^{125}I-2 was obtained in modest isolated radiochemical yields (*ca.* 20%) with high radiochemical purities by both methods. The former gave specific activities of 0.2—0.6 kBq/μmol, whereas the latter approach achieved specific activities of more than 0.14 GBq/μmol. On attempting to prepare an injectable formulation for ^{125}I-2 with high specific activity, its radiochemical purities dropped to about 60—70%. Unlabeled analog 2 was found to have lipophilic and antioxidant properties similar to edaravone. Intravenous injection of ^{125}I-2 with low specific radioactivity into normal mice showed signs of distribution profiles similar to reported results for ^{14}C-labeled edaravone in normal rats.

Key words edaravone; radioiodine; labeling; stability; biodistribution; mouse

Recent studies indicate that reactive oxygen species (ROS), such as hydroxyl radical and hydrogen peroxide, are generated in a normal physiological process, and that the imbalance between the antioxidant defense system and excessive levels of ROS in cellular systems is involved in the initiation and progression of various diseases and disorders.[1—5] Various chemical probes of several methods have been developed to measure ROS in biological systems and the most common approach is the measurement of the levels of trapped molecules formed as a result of interception of ROS by probe molecules.[6,7] Potential problems in detection and quantification of ROS by methods currently available have been reviewed,[8,9] in which the difficulties of precisely analyzing ROS-expression in the whole body are discussed, because of the low concentration of ROS formed in tissues and interference by the presence of endogenous reductants. Nevertheless, *in vivo* imaging technologies that allow visualization of localized production and accumulation of ROS in a living system provide useful information on the pathophysiological status of the living body, and they have attracted a great deal of interest.

Of several biological imaging methods, nuclear imaging technology, such as positron emission tomography or single photon emission computed tomography using radiolabeled molecules, is well known for its ability to assess molecular pathways *in vivo* in both pre-clinical and clinical studies with very high sensitivity.[10] The first reported work in developing radiotracers for free radical imaging *in vivo* includes ^{125}I-labeled analogs of α-*p*-hydroxy-*m*-iodophenyl-*N*-*t*-butylnitrone[11] and α-*p*-iodophenyl-*N*-*tert*-butyl-nitrone,[12] based on the chemical characteristic that free radicals can be reacted with nitrone-based compounds at the nitrone-double bond to form organic radical adducts, but none of these proved to be useful as *in vivo* probes because of their limited free radical specificity. An ^{18}F-labeled analog of *N*-*t*-butylnitrone[13] or ethylmethylthiourea compound[14] has also been prepared, but with no information on its *in vivo* behavior. Thus, no appropriate radiotracers for nuclear imaging technology are currently available for detecting free radicals *in vivo*.



Edaravone (**1**) OPB

Fig. 1. Edaravone (**1**) and Its Radical-Induced Oxidation Product (OPB)

3-Methyl-1-phenyl-2-pyrazolin-5-one (edaravone, **1**, Fig. 1), known as a potent free radical scavenger, has been used clinically for the treatment of cerebral infarction in humans to prevent ischemic reperfusion injury[15—17] and has also been reported to be effective for myocardial and hepatic ischemia as well.[18,19] Biochemical and chemical studies have found that the free radical scavenging reaction of edaravone (**1**) with free radical species produces mainly 2-oxo-3-(phenylhydrazono)butanoic acid (OPB) with a more hydrophilic character as a stable oxidative product.[17,20,21] In addition, metabolite studies have indicated that edaravone, after intravenous administration in rats and humans, is exclusively metabolized into its glucuronide and/or sulfate conjugates which are excreted into the urine *via* the kidney,[22,23] except for oxidation process according to its radical scavenging functions. Such *in vivo* behavior of edaravone thus appeared to have favorable properties as a lead molecule for the development of radiotracers of the metabolic trapping type suitable for detecting free radicals *in vivo*. In order to investigate the possibility of developing new radiotracers for probing free radical expression *in vivo*, we were interested in iodinated analogs of edaravone where an iodine atom was introduced at the *meta* position relative to the phenyl ring of the molecule due to the expected stability against *in vivo* deiodination.[24] Here we describe the synthesis and some physicochemical properties of 1-(3′-iodophenyl)-3-methy-2-pyra-

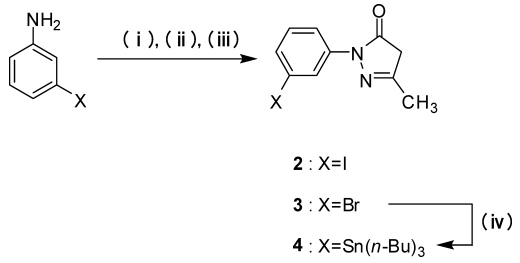
* To whom correspondence should be addressed. e-mail: maeda@phar.kyushu-u.ac.jp; mukai@phar.kyushu-u.ac.jp

zolin-5-one (**2**), the radiosynthesis of the ^{125}I-labeled analog (^{125}I-**2**) with low and high specific radioactivity, and its preliminary biodistribution in normal mice.

Results and Discussion

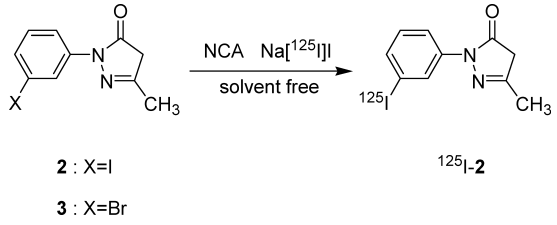
According to a similar literature procedure,[25—27] 1-(3′-iodophenyl)-3-methy-2-pyrazolin-5-one (**2**) was prepared in three steps from 3-iodoaniline, involving diazotization in hydrochloride–acetic acid, followed by reduction with stannous chloride and cyclization with ethyl acetoacetate in EtOH, in 49% overall yield of the three-step conversion, as presented in Chart 1. It was important to carry out this under a stream of argon gas for the reduction stage. Cyclization with ethyl acetoacetate to form a pyrazolone ring proceeded sluggishly in our initial experiments, giving low yields of the required product. After many attempts, the addition of two equivalents of ethyl acetoacetate in small portions to a solution of the hydrazine compound in MeOH over 10 min was found to give much better results, and the formation of the by-products became significant when ethyl acetoacetate was added rapidly. 1-(3′-Bromophenyl)-3-methy-2-pyrazolin-5-one (**3**), for use as a precursor of radioiodination described below, was also prepared from 3-bromoaniline as similarly described for iodoedaravone (**2**), but it gave a complex mixture of products and only a very low yield of **3** as a crystalline compound due to the technical difficulty in chromatographic purification to obtain a product of satisfactory purity and instability for the hydrazine base. The tributylstannylated precursor (**4**) for use in the electrophilic labeling approach was also prepared in 23% yield by refluxing a solution of **2** and bistributyltin in dry toluene in the presence of a palladium catalyst.

The log partition coefficient (1-octanol/67 mM phosphate buffer, pH=7.4) of **2**, determined by the shake-flask technique and HPLC quantification, was found to be 1.35±0.17 whereas that of edaravone (**1**) was 1.05±0.18, indicating that



(i) $NaNO_2$, HCl–AcOH; (ii) $SnCl_2$, HCl; (iii) CH_3COCH_2COOEt, EtOH; (iv) Bu_6Sn_2, $Pd(Ph_3)_4$, toluene.

Chart 1



Radiosynthesis of 1-(3′-[^{125}I]iodophenyl)-3-methy-2-pyrazolin-5-one (^{125}I-**2**) by exchange of no-carrier-added Na[^{125}I]I for iodinated and brominated edaravone (**2**, **3**) under solvent-free conditions.

Chart 2

2 is slightly more lipophilic than edaravone itself, as expected. It is thought that edaravone (**1**) can be distributed between the aqueous and lipid phases,[29,30] thereby preventing lipid peroxidation in both phases, and a lipophilic substituent, such as the iodine atom, may be beneficial to the lipidic peroxidation inhibitory activity of edaravone, as suggested.[27] Next, the hydrogen-donating capacity by DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity using a simple UV spectroscopic method,[31] and the one-electron oxidation potential (Epa) by the cyclic voltammetry technique, which are prerequisites for further investigation, were examined for **2** in comparison with that for **1**. It can be seen from Table 1 that **2** exhibits almost the same rate constant as edaravone **1** for the reaction with DPPH in a mixture of EtOH and Tris buffer (pH=7.4). The oxidation potentials were measured in a basic aqueous solution at a pH range of 10.0—10.1 because of the poor solubility of compound (**2**) in acidic and neutral solutions. Iodoedaravone (**2**) had an oxidation potential of 372 mV (*vs.* Ag^+/AgCl), which showed about a 50-mV lower value compared to edaravone itself as shown in Table 1. These results suggest that introduction of an iodine atom at *meta*-position of the phenyl ring of edaravone molecule has a little effect on both lipophilicity and antioxidant activity.

Iodine-125 was selected as the radiolabel for our radiotracer development work because the long half-life (60 d) and low radiation energy of this radioisotope simplify radiosynthesis. A number of radioiodination methods have been developed for the radiolabeling of biological and medical interests[34] and, among them, a solvent-free procedure for the radioiodination of aromatic compounds has often been employed when the compound to be labeled is stable at its melting point. Our initial approach to the synthesis of ^{125}I-**2** was *via* a conventional exchange reaction, under solvent-free conditions, of nonradioactive **2** with no-carrier-added (NCA) Na[^{125}I]I which had been previously dried by azeotropic evaporation with dry CH_3CN under a stream of nitrogen at room temperature. During the labeling reaction, which required a high temperature of 140 °C in a closed vessel, the label incorporation began to occur from 10—15 min, followed by a gradual incorporation by additional 15—20 min, but either prolonged heating over 30 min or temperatures higher than 140 °C led to the decomposition of the labeled product formed. An average isolated radiochemical yield of 23% with specific radioactivities between 0.2—0.6 kBq/μmol and high radiochemical purity (>98%) was obtained after reversed-phase HPLC purification (Fig. 2).

Specific activity appears to influence the *in vivo* radical scavenging ability of the radiotracer, since the presence of the carrier molecule may have a negative effect on the concentration of radioactivity in targeted tissues. In order to achieve high specific radioactivity in the preparation of ^{125}I-**2**, the electrophilic demetallation labeling method using an organotin precursor was attempted.[35] The stannylated precursor (**4**) was subjected to a radioiodo-destannylation reaction with NCA Na[^{125}I]I under classical reaction conditions, involving a combination of H_2O_2–AcOH, chloramine-T or *N*-chlorosuccinimide, but no detectable ^{125}I-**2** was observed, even with an excess amount of oxidizing agents or an increase in reaction time. It is most likely that the observed lack of any response of **4** toward the radioiodine-destannyla-

Table 1. The Lipophilicity, DPPH Scavenging Activity and Oxidation Potential

	log $P^{a)}$	Antioxidant activity	
		DPPH scavenging activity$^{b)}$	E_{pa} (mV)$^{c)}$
Iodoedaravone (**2**)	1.35±0.17	0.264±0.010/s	372±1.5
Edaravone (**1**)	1.05±0.18	0.237±0.007/s	422±5.0
	0.78$^{d)}$		483$^{e)}$

a) Expressed as log *P* (1-octanol–67 mM phosphate buffer pH=7.4). Values represent the mean±S.D. of three experiments. *b*) Reducing activity of 2.0×10^{-4} M antioxidant against 2,2-diphenyl-1-picrylhydrazyl (DPPH) in EtOH–50 mM Tris–buffer (pH=7.4)=3 : 2. Rate constants were obtained from the linear portion of the decrease in optical density at 517 nm. Values represent the mean±S.D. of five experiments. *c*) Conditions for measurement: 0.025 mmol samples in aqueous solution (pH=10.0—10.1); the oxidation potentials were expressed *versus* Ag^+/AgCl and the values represent the mean±S.D. of three experiments. *d*) Taken from ref. 28. *e*) Taken from refs. 32, 33.

tion reaction was caused by the antioxidant property of its pyrazolone ring.

Therefore, we examined an alternative method which does not utilize an electrophilic iodine source. Thus, heating bromoedaravone (**3**) with NCA Na[^{125}I]I at 140 °C for 60 min in a solvent-free procedure provided ^{125}I-**2** in an average isolated radiochemical yield of 18% after HPLC separation (Fig. 2), reflecting lower reactivity of **3** in regard to the displacement reaction. The obtained ^{125}I-**2** had >98% radiochemical purity without contamination from the labeling precursor and the specific radioactivity was estimated to be in excess of 0.14 GBq/μmol based on the UV absorbance detection limit of the HPLC system used. Thus, use of bromoedaravone (**3**) for the interhalogen exchange reaction produced ^{125}I-**2** with a much higher specific activity compared to that obtained by the isotopic exchange method.

After removal of the solvent (MeOH–H_2O=45 : 55 (v/v)

Table 2. Distribution of Radioactivity in Tissues of Normal Mice Following Intravenous Administration of 1-(3′-[^{125}I]Iodophenyl)-3-methy-2-pyrazolin-5-one (^{125}I-**2**), Obtained by the Isotopic Exchange Method

Organ	% Injected dose per gram (n=3—5), mean±S.D.				
	5 min	30 min	60 min	6 h	24 h
Blood	9.21±0.58	3.26±0.40	2.01±0.35	0.36±0.07	0.07±0.02
Spleen	2.06±0.14	0.88±0.15	0.85±0.08	0.36±0.09	0.14±0.04
Pancreas	2.77±0.52	1.01±0.32	0.44±0.16	0.14±0.03	0.04±0.01
Stomach	2.36±0.39	0.88±0.16	0.72±0.12	0.42±0.22	0.09±0.01
S. content$^{a)}$	0.98±0.10	0.50±0.34	0.44±0.13	0.67±1.15	0.04±0.02
Small intestine	7.39±1.81	1.47±0.11	1.07±0.27	0.22±0.08	0.08±0.04
S. i. content$^{b)}$	20.06±5.30	1.90±0.68	1.02±0.44	0.26±0.15	0.07±0.02
Large intestine	2.31±0.44	0.64±0.11	0.86±0.24	0.23±0.07	0.07±0.03
L. i. content$^{c)}$	0.42±0.21	0.57±0.41	5.28±3.79	2.08±0.67	0.18±0.10
Kidney	26.03±6.65	3.15±0.30	1.95±0.53	0.56±0.09	0.28±0.04
Liver	10.35±2.43	2.38±0.24	1.28±0.20	0.24±0.02	0.05±0.02
Heart	2.83±0.25	1.62±0.49	0.81±0.09	0.18±0.04	0.05±0.01
Lung	6.26±0.42	2.44±0.26	1.50±0.22	0.60±0.10	0.27±0.05
Muscle	1.63±0.49	0.60±0.28	0.79±0.50	0.22±0.08	0.09±0.06
Brain	0.48±0.04	0.20±0.04	0.10±0.03	0.03±0.01	0.02±0.01
Bone	2.10±0.31	1.19±0.75	0.68±0.20	0.16±0.03	0.21±0.15
Artery	10.04±1.15	4.28±0.87	3.11±0.10	1.19±0.26	1.07±0.48
Thyroid$^{d)}$	0.12±0.03	0.11±0.07	0.09±0.03	0.14±0.02	0.33±0.11

a) s.content=stomach content, *b*) s.i.content=small intestine content, *c*) l.i.content=large intestine content, *d*) expressed as % of injected dose/organ.

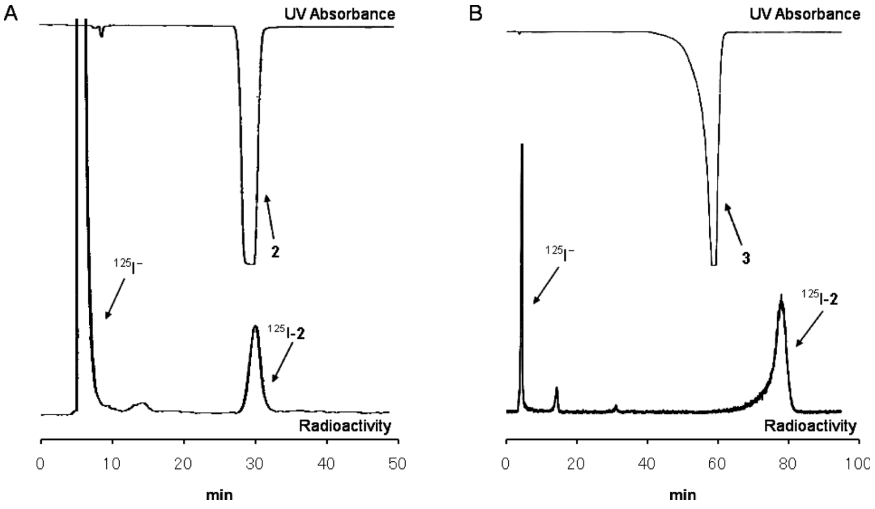


Fig. 2. Preparative HPLC-Chromatograms for Isolation of ^{125}I-**2** from the Reaction Mixtures on a Reversed-Phase Column

(A) Isotopic exchange between iodinated edaravone (**2**) and NCA Na[^{125}I]I; (B) nonisotopic exchange between brominated edaravone (**3**) and NCA Na[^{125}I]I.

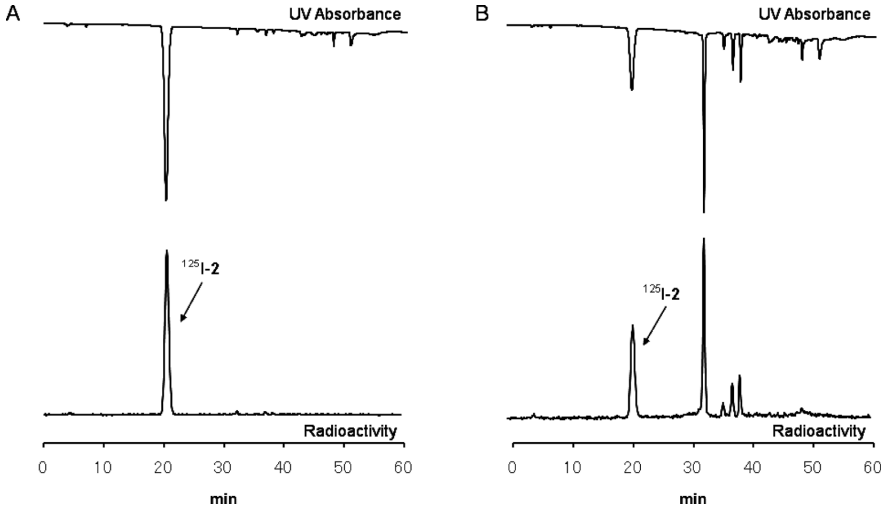


Fig. 3. Typical HPLC Chromatograms of the Formulated Solution after Removal of the HPLC Solvent of the Fractions Containing Low Specific Activity ^{125}I-**2**; $t=0$ h (A) and $t=3$ h (B) after Storage at 37 °C

with 0.1% CF_3COOH in both methanol and water) of the HPLC fractions containing the isolated radioactive compound under reduced pressure, the residue was redissolved in 1% Tween-80-physiological saline containing 5% EtOH as an injectable solution. The formulated solution of ^{125}I-**2** with low specific activity remained stable up to 6 h at 0 °C, but the radiochemical purity dropped off to 80% in 1 h and 40% in 3 h after storage at 37 °C. HPLC analysis of the formulated solution, as shown in Fig. 3, revealed the presence of at least four radioactive peaks newly produced, in a form more lipophilic than the parent tracer, as judged from their longer retention times by HPLC. The chemical forms of these radioactivity peaks are of unknown identity at present, although neither HPLC nor TLC analysis gave any indication of the formation of free ^{125}I-iodide ion. In contrast to the low specific activity ^{125}I-**2**, we found that, on attempting to remove the HPLC solvent containing ^{125}I-**2** with high specific activity, the formation of new radioactive substances was frequently occurring. A consequence was that the radiochemical purity in the formulated solution decreased to a level of 60—70%. Additional storage at 37 °C promoted a further decrease of ^{125}I-**2** and after 6 h no radioactivity remained as the parent ^{125}I-**2**. HPLC analysis gave the same HPLC peaks of radioactive substances as observed after storage at 37 °C of the formulated solution of of the ^{125}I-**2** with low specific radioactivity. The lability observed in the formulation step of ^{125}I-**2** with high specific radioactivity appears to be related to both self-absorbed radiation and chemical instability of the compound itself. In the present study we have not been able to develop a suitable method for stabilization of the high specific activity ^{125}I-**2** after preparation, and therefore, in this work, the *in vivo* application of this agent was not carried out.

Tissue biodistribution of ^{125}I-**2** with low specific activity was thus evaluated in normal mice by the dissection procedure and the results are expressed as % injected dose (ID)/g of tissue or %ID/organ as shown in Table 2. ^{125}I-**2** demonstrated low initial blood activity (3.26±0.40%ID/g at 30 min after injection) and relatively fast clearance after 24 h (0.07± 0.02%ID/g). Early renal and hepatic uptake was higher than that in other tissues except the arteries, but the radioactivity from these organs washed out with time, approaching the levels seen in other tissues. The accumulation of radioactivity in the thyroid was low (0.14±0.02%ID/organ at 6 h) and remained constant at all points of the study, confirming the expected stability of the iodophenyl group in regard to *in vivo* deiodination. The presence of a substantial amount of radioactivity in the small and large intestines, and also intestinal content seem to be an indication of extensive biliary excretions of radioactivity. ^{125}I-**2** showed very little uptake in the mouse brain even at 5 min after injection, thus demonstrating limited permeability through the blood–brain barrier in the normal mouse brain, as observed for 'phenyl ring'-^{14}C-labeled edaravone (specific activity: 4.5 GBq/mmol) in rats.[23] ^{125}I-**2** had a relatively higher radioactivity concentration in the aorta tissue relative to other tissues at 30 and 60 min after injection. Similar uptake and retention of radioactivity in aorta tissue was reported in a biodistribution study of rats injected with ^{14}C-labeled edaravone by Komatsu *et al.*,[36] in which it was proposed as a chemical trap for ^{14}C-labeled edaravone with free radical species existing in this tissue, although it is largely speculation in the absence of detailed studies. Thus, from the tissue biodistribution in normal mice, a general clearance of the radioactivity over time in all organs was found, qualitatively similar to that seen with ^{14}C-labeled edaravone in rats,[23] in spite of the large differences in specific activities of these agents.

Conclusion

In the present study, two radiolabeling methods *via* isotopic exchange and interhalogen exchange were evaluated to synthesize 1-(3′-[^{125}I]iodophenyl)-3-methy-2-pyrazolin-5-one (^{125}I-**2**) with regard to radiochemical yield, radiochemical purity, and specific activity in order to develop a radiotracer for probing free radicals *in vivo*. The radiosynthesis provided radiochemically pure ^{125}I-**2** in reasonable quantities by both methods, although further optimization is possible. The biodistribution showed significant elimination of radioactivity from normal tissues and relatively low levels of circulating radioactivity in the blood pool during the 6—24 h. Solution stability studies demonstrated that ^{125}I-**2** has the disadvantage of inherent instability in an aqueous solution. Fur-

ther study will focus on the development of formulation procedure, capable of maintaining radiochemical purity of ^{125}I-**2** with high specific radioactivity, and it is also necessary to address the labeled products resulting from the exposure of this radiotracer to tissues *in vitro* and *in vivo*.

Experimental

Edaravone was supplied by Mitsubishi Pharma Co. (Tokyo, Japan) and had a purity of >95%. Chemical reagents and solvents were of commercial quality and were used without further purification unless otherwise noted. ^{1}H-NMR spectra were obtained on a Varian Unity 400 (400 MHz) and are referenced to tetramethylsilane (TMS) (δ=0 ppm). Infrared (IR) spectra were recorded with a Shimadzu FTIR-8400 spectrometer and mass spectra were obtained with a JEOL JMS DX-610 (FAB Mass) or an Applied Biosystems Mariner System 5299 spectrometer electrospray ionization-mass spectra (ESI-MS). All melting points were determined on a Yanaco melting point apparatus (Yanagimoto Ind. Co., Japan) and are uncorrected. Column chromatography was performed on Silica gel 60N (63—210 mesh, Kanto Chemical Co., Inc., Japan) and the progress of the reaction was monitored by TLC on Silica gel 60F 254 plates (Merck, Germany), and the spots were visualized with UV light or by spraying with 5% alcoholic molybdophosphoric acid. In the synthetic procedures, the organic extracts were routinely dried over anhydrous Na_2SO_4 and evaporated with a rotary evaporator under reduced pressure. HPLC was done using a Shimadzu Liquid Chromatograph System (SPD-10A) (COSMOSIL 5C18-PAQ column, 250×4.6 mm, Nacalai Tesque, Japan) and by monitoring the radioactivity (NaI(Tl) detector) as well as UV absorption (at 254 nm). The radioactivity was quantified with an auto-well gamma counter (ARC-605 II, Aloka, Japan). Analysis of the radioactivity of the TLC plates was performed by cutting into 0.5 cm-wide strips and counting their radioactivity with a gamma-well counter. Identity of the labeled compound, specific radioactivity and radiochemical purity were determined by the same HPLC system (COSMOSIL 5C18-AR-II, 250×4.6 mm, Nacalai Tesque, Japan) or radio TLC. No-carrier-added Na[^{125}I]iodide (0.04 M NaOH solution; 185 MBq, >600 GBq/mg) was purchased from American Radiolabeled Chemicals Inc. Animal experiments were carried out in accordance with our institutional guidelines and were approved by the Animal Care and Use Committee, Kyushu University.

Synthesis. 1-(3′-Iodophenyl)-3-methy-2-pyrazolin-5-one (2) A solution of 3-iodoaniline (438 mg, 2 mmol) in acetic acid (1.64 ml) and concentrated HCl (4.78 ml) was treated with an ice-cold solution of $NaNO_2$ (207 mg, 3 mmol) in H_2O (776 μl). The mixture was stirred for 1 h at this temperature. Stannous chloride dihydrate (1.354 g, 6 mmol) was dissolved in concentrated HCl (925.4 μl) and the solution was cooled to 0 °C under an argon atomosphere. To the diazonium mixture produced was slowly added a solution of the stannous chloride over a period of 2 h under an argon atmosphere, while the temperature was always kept at 0 °C. The mixture was made basic by the addition of an excess of 10% NaOH and extracted several times with ether. The combined organic extracts were dried and evaporated to dryness. The reddish brown residue was dissolved in EtOH (5 ml) and to the mixture was slowly added ethyl acetoacetate (234 mg, 1.8 mmol) over a period of 10 min at 50 °C under stirring. The mixture was further heated under reflux for 3 h. After removal of the solvent under vacuum, the residue was chromatographed on silica gel (chloroform–hexane=1 : 3) to give **2** as an orange solid (296 mg, 49%), mp 128—132 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.21 (s, 3H), 3.47 (s, 2H), 7.10 (t, 1H, J=16.1 Hz), 7.51 (d, 1H, J=8.1 Hz), 7.90 (d, 1H, J=10.3 Hz), 8.24 (t, 1H, J=3.6 Hz); IR (KBr) cm^{-1}: 1716, 1582, 1474; FAB-MS (m/z): 301.08 (M+H)$^+$; ESI-HR-MS (m/z): Calcd for $C_{10}H_{10}N_2OI$ (M+H) 300.9832, Found 300.9847.

1-(3′-Tri-*n*-butylstannylphenyl)-3-methy-2-pyrazolin-5-one (4) 1-(3′-Iodophenyl)-3-methy-2-pyrazolin-5-one (**2**) (30 mg, 0.1 mmol) was dissolved in dry toluene (30 ml) which had been degassed by bubbling argon gas through it for 5 min. Tetrakis(triphenylphosphine)palladium(0) (11.55 mg, 0.01 mmol) and bis(tri-*n*-butyl)tin (145 mg, 0.25 mmol) were successively added. The reaction mixture was heated under reflux for 3 h. The black reaction mixture was diluted with dichloromethane and filtered, and the solvent was removed under vacuum. Purification was carried out using column chromatography on silica gel (chloroform–hexane=1 : 1) to give compound (**4**) as a yellow-brown oil (10.8 mg, 23%). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (t, 9H, J=7.3 Hz), 1.07 (t, 6H, J=8.2 Hz), 1.33 (m, 6H), 1.53 (m, 6H), 2.19 (s, 3H), 3.41 (s, 2H), 7.34 (t, 2H, J=7.7 Hz), 7.77 (d, 1H, J=8.1 Hz), 7.92 (d, 1H, J=2.2 Hz); IR (neat) cm^{-1}: 2957, 2927,1725, 1580; ESI-MS (m/z): 465.1 (M+H).

1-(3′-Bromophenyl)-3-methy-2-pyrazolin-5-one (3) Compound (**3**) was obtained from 3-bromoaniline (688 mg), by diazotization in hydrochloride–acetic acid, followed by reduction with stannous chloride dihydrate (2.7 g) and cyclization with ethyl acetoacetate (182 mg) in EtOH, according to the procedure described for **2**. Removal of the solvent under vacuum gave a thick oily residue, which was purified by repeated column chromatography on silica gel(chloroform–hexane=1 : 3) to give **3** as an orange solid (38 mg, 4%), mp 132—134 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.32 (s, 3H), 3.58 (s, 2H), 7.33 (m, 1H), 7.43 (d, 1H, J=8.0 Hz), 7.54 (d, 1H, J=8.0 Hz), 7.65 (s, 1H); IR (KBr) cm^{-1}: 1684, 1626, 1578, 1481; ESI-HR-MS (m/z): Calcd for $C_{10}H_{10}N_2OBr$ (M+H) 252.9967, Found 252.9971; Calcd for $C_{10}H_{11}N_2OBr$ (M+3H) 254.9952, Found 254.9951.

Lipophilicity The partition coefficients of **2** and edaravone (**1**) were determined at room temperature from the distribution of the compounds between a mixture of 1-octanol (2.5 ml) and a 67 mM phosphate buffer (pH=7.4, 2.5 ml). The mixture was vortexed for 3 min and was left to stand for 10 min. This procedure was repeated three times, followed by centrifugation at 3000×***g*** for 5 min. The concentration of the compound in both phases before and after the shaking were analyzed by HPLC (column; Nacalai Tesque COSMOSIL 5C18-AR-II, 4.6×250 mm, mobile phase: MeOH–0.1% aqueous CF_3COOH 72 : 28, v/v, flow rate: 0.5 ml/min, UV: 254 nm, retention time: 13.5 min for **2** and 7.5 min for edaravone). The log P value represents the mean±S.D. of three independent experiments.

DPPH Radical-Scavenging Assay The radical scavenging activity against the DPPH radical was determined according to the method described in the literature, with slight modifications.[31] Briefly, changes in the absorbance at 517 nm due to the scavenging of the DPPH radical was continuously monitored with a spectrophotometer (Hitachi U-2810 spectrometer) immediately after mixing (1.5 ml) a freshly prepared 2.0×10^{-4} M solution of DPPH in a mixture of a 50 mM Tris–HCl buffer (pH 7.4) and EtOH (2 : 3, v/v), and a 2.0×10^{-4} M solution of **2** or edaravone (**1**) in the same solvent system at 25 °C. The first-order of rate constant for the disappearance of the DPPH color at 517 nm was calculated from the slope of the linear portion of the disappearance curves, and the result was expressed as the mean±S.D. of five determinations.

Cyclic Voltammetry Cyclic voltammograms were measured using BAS 100B/W (CV-50W) version 2 potentiometer consisting of platinum electrodes as working and counter electrodes. Potentials were measured relative to the Ag^+/AgCl reference electrode. An internal reference (potassium ferricyanide) was used as well. Edaravone (4.35 mg, 0.025 mmol) or **2** (7.5 mg, 0.025 mmol) was dissolved in 2 ml of 1 M NaOH and then the solutions were adjusted to pH 10.0—10.1 with 1 M HCl (final concentrations: 6.4 mM for edaravone and 6.6 mM for **2**). Oxygen was removed by purging the solution with argon gas and a continuous stream of this gas was passed over the solutions during the measurements. Cyclic voltammograms were obtained at a scan rate of 50 mV/s. The values of the oxidation potential represent the mean±S.D. of three independent measurements.

Radiochemistry. 1-(3′-[^{125}I]Iodophenyl)-3-methy-2-pyrazolin-5-one (^{125}I-2) (A) Iodine-Radioiodine Exchange: No-carrier-added Na[^{125}I]I (0.37—7.2 MBq) was transferred to a reaction vial and to this was added dry CH_3CN (30 μl), followed by evaporation to dryness under a stream of nitrogen at room temperature. To the residue was then added a solution of **2** (50—500 μg) in dry CH_3CN (500 μl) and evaporation was repeated. The vial was flushed with a stream of nitrogen for a few seconds, tightly sealed and heated at 140 °C for 30 min. The entire content of the reaction vessel was dissolved in methanol (100 μl) and was injected onto the HPLC column (Nacalai Tesque COSMOSIL 5C18-PAQ 4.5×250 mm, MeOH/H_2O=45/55 (v/v) with 0.1% CF_3COOH in both methanol and water which were bubbled with nitrogen gas beforehand, flow rate 0.8 ml/min). The product fraction was collected after a retention time of 30.1 min. The isolated radiochemical yield of ^{125}I-**2** ranged from 6.6 to 41.3% (average of 23%) in a total preparation time of 60 min. The specific activities of the obtained ^{125}I-**2** varied from 0.2 to 0.6 kBq/μmol, as determined by direct measurement of the HPLC eluate using a UV detector. Quality control HPLC showed a single radioactive peak with the corresponding carrier, suggesting that the preparations were more than 98% chemically and radiochemically pure. The product was formulated by evaporating the fraction collected from HPLC under reduced pressure, and dissolving in 1% Tween-80-physiological saline containing 5% EtOH.

(B) Bromine-Radioiodine Exchange: To no-carrier-added Na[^{125}I]I (0.37—3.7 MBq), which had been dried as described above, was added a solution of **4** (100—500 μg) in dry CH_3CN (500 μl) and evaporation was carried out under a stream of nitrogen at room temperature. The vial was flushed with a stream of nitrogen for a few seconds, then tightly sealed and heated at 140 °C for 60 min. The entire content of the reaction vessel was

dissolved in methanol (100 μl) and was injected onto the HPLC column (Nacalai Tesque COSMOSIL 5C18-PAQ 4.5×250 mm, MeOH/H_2O=40 : 60 (v/v) with 0.1% CF_3COOH in both methanol and water which were bubbled with nitrogen gas before hand, flow rate of 0.8 ml/min). The product fraction was collected at a retention time of 80.1 min. The isolated radiochemical yield of ^{125}I-**2** ranged from 3.6 to 32.8% (average of 18%) in a total preparation time of 150 min. The specific radioactivity was estimated to be in excess of 0.14 GBq/μmol based on the UV absorbance detection limit of the HPLC system used. Quality control HPLC showed a single radioactive peak and no UV peaks, suggesting that the preparations were more than 98% chemically and radiochemically pure. For formulation, the solvent of the HPLC fraction was evaporated under reduced pressure and the radioactivity was dissolved in 1% Tween-80-physiological saline containing 5% EtOH.

Solution Stability The formulated solution of ^{125}I-**2** was allowed to stand at 0 °C and 37 °C. At various intervals from zero to 6 h, the samples were withdrawn and analyzed for stability by analytical HPLC and radio-TLC. HPLC analysis was performed using a COSMOSIL 5C18-AR-II column (250×4.6 mm, Nacalai Tesque). The column was eluted with a gradient of 0.1% CF_3COOH in MeOH (A) and 0.1% CF_3COOH in H_2O (B) at 0.7 ml/min. The percentage of (A) was increased linearly to 100% over 45 min and maintained at that value for another 15 min. Under these conditions ^{125}I-**2** was eluted at about 20.4 min. TLC analysis was performed under a stream of nitrogen gas using Merck TLC aluminium backed sheets Silicagel 60 RF-18 F_{254S}; developing solvent; MeOH–H_2O–CH_3COOH=50 : 20 : 1 with 0.02% ascorbic acid (w/v), which was bubbled with nitrogen gas beforehand; *Rf*=0.00—0.03 for $^{125}I^-$ ion and *Rf*=0.24—0.27 for ^{125}I-**2**.

Tissue Distribution Studies in Normal Rats Normal male ICR mice (6 weeks old, weighing 30—35 g), deprived of food 18 h prior to and during the course of experiment, with free access to water, were used in this investigation. The animals were intravenously injected through the tail vein with a solution of ^{125}I-**2** (9.0—11.1 kBq, 100 μl, specific activity 0.2—0.6 kBq/μmol) in 1% Tween-80-physiological saline containing 5% EtOH, immediately after HPLC purification. At pre-designated time points, the mice were sacrificed by exsanguination under ether anesthesia. Blood was collected by heart puncture and the tissues were harvested. Abdominal aorta and thoracaorta samples were also collected. The radioactivity in the samples was measured in a gamma counter (ALOKA, ARC-370) and the tissues were weighed. The tissue uptake of the radioactivity was expressed as a percentage of the injected dose per gram of tissue (%ID/g of tissue) or a percentage of the injected dose per organ (%ID/organ).

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